Docket No.: RUJ-001CNCPRCE2
(PATENT)

SEP 2 8 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Daniel Hawiger *et al.*

Application No.: 09/925,284	Confirmation No.: 2660
Filed: August 9, 2001	Art Unit: 1644
For: ENHANCED ANTIGEN DELIVERY AND MODULATION OF THE IMMUNE RESPONSE THEREFROM	Examiner: R. B. Schwadron

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SECOND REPLACEMENT APPEAL BRIEF

As indicated in the Notice of Appeal filed on June 28, 2006, Appellants hereby appeal the final decision of the Examiner in the above-identified application rejecting the subject matter of the pending claims. For the reasons set forth in this Appeal Brief, Appellants respectfully request the Board of Patent Appeals and Interferences to reverse the Examiner's final rejection of the claimed subject matter. As Appellants are filing this Appeal Brief within the one month time period for responding to the Notification of Non-Compliant Appeal Brief mailed on August 28, 2007 (due for response on September 28, 2007), no fees are believed to be due. However, if this is in error, authorization is hereby given to charge any fees to deposit account number 12-0080.

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I.   REAL PARTY IN INTEREST

The real parties in interest in the above-identified application are Rockefeller University, the assignee of the application, and Celldex Therapeutics, Inc., the licensee of the application.

II.   RELATED APPEALS AND INTERFERENCES

An Appeal Brief was filed in the parent application, U.S.S.N.: 09/586,704, having a filing date of June 5, 2000. No related interferences are known to Appellants, which will directly affect, or be directly affected by, or have a bearing on the Board's decision in the pending appeal.

III.   STATUS OF CLAIMS

Claims 1-21 are pending in this application. Claims 1-5 and 10-12 have been withdrawn from consideration.

Claims 6-9 and 13-21 are on appeal and are set forth in the Claims Appendix (Appendix A).

IV.   STATUS OF THE AMENDMENTS

A Notice of Appeal was filed June 28, 2006. All prior amendments have been entered.

V.   SUMMARY OF CLAIMED SUBJECT MATTER

<u>Claims 6-9</u>

The claims on appeal are drawn to methods for enhancing tolerance to a preselected antigen in a mammal, comprising exposing *ex vivo* or *in vivo* dendritic cells from the mammal to a vaccine conjugate that comprises the preselected antigen covalently bound to an anti-human DEC-205 antibody, or an anti-murine DEC-205 antibody that binds to human DEC-205, under conditions that promote dendritic cell quiescence, wherein the human DEC-205 protein comprises the amino acid sequence of SEQ ID NO: 7. The amino acid sequence of SEQ ID NO:7 corresponds to a partial (C-terminal) sequence of human DEC-205 (see, for example, page 11, lines 1-16; page 18,

lines 5-10; page 25, line 16 through page 26, line 5; page 27, lines 13-17 of the specification as originally filed).

Claims 13-17

The claims on appeal are further drawn to methods for enhancing tolerance to a preselected antigen for which tolerance is desired in a mammal, comprising exposing *ex vivo* or *in vivo* dendritic cells from said mammal to a conjugate comprising said preselected antigen covalently bound to an anti-human DEC-205 antibody (claim 13) or anti-murine DEC-205 antibody (claim 14), wherein the antibody is reactive with an amino acid sequence as set forth in SEQ ID NO: 7. As noted above, the amino acid sequence of SEQ ID NO:7 corresponds to a partial (C-terminal) sequence of human DEC-205 (see, for example, page 11, lines 1-16; page 18, lines 5-10; page 25, line 16 through page 26, line 5; page 27, lines 13-17 of the specification as originally filed).

Claims 18-21

The claims on appeal are also drawn to methods for enhancing tolerance to a preselected antigen in a mammal comprising exposing *ex vivo* or *in vivo* dendritic cells from the mammal to a conjugate comprising the preselected antigen bound to an anti-mouse DEC-205 antibody that cross-reacts with human DEC-205, under conditions that promote dendritic cell quiescence, wherein the mouse DEC-205 protein comprises the amino acid sequence of SEQ ID NO: 10. The amino acid sequence of SEQ ID NO:10 corresponds to the full-length sequence of mouse DEC-205 (see, for example, page 11, lines 1-16; page 18, lines 5-10; page 25, line 16 through page 26, line 5; page 27, lines 13-17 of the specification as originally filed).

Also, see the full length mouse DEC-205 sequence (SEQ ID NO: 3) in the parent application, U.S.S.N. 09/586,704, which was incorporated by reference in its entirety in the present application, U.S.S.N. 09/925,284. Please also see the Substitute Sequence Listing submitted in the present application on December 22, 2005, which identifies the full length mouse DEC-205 sequence as SEQ ID NO: 10.

VI.   GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Appellants present the following issue for review:

1.   Whether claims 6-9 and 13-21 are properly rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement.

2.   Whether claims 6-9 and 13-17 are properly rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement.

VII.   ARGUMENTS

A. Summary of Examiner's Rejection of Claims 6-9 and 13-21 Under 35 U.S.C. § 112, First Paragraph, as Failing to Comply with the Written Description Requirement

The Examiner has rejected claims 6-9 and 13-21 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. In particular, the Examiner asserts that the specification does not provide adequate written description for the claimed invention because, while the specification discloses the full length sequence of murine DEC-205 protein, it only discloses a partial sequence for human DEC-205. The Examiner asserts that, because human DEC-205 is approximately 1800 amino acids in length, the recitation in the claim of a 30 or 25 amino acid sequence derived from human DEC-205 does not provide adequate written description of a molecule that is almost 1800 amino acids in length. The Examiner further asserts that the claims encompass antibodies that bind any immunogenic epitope on the approximately 1775 undisclosed amino acids of DEC 205, and that the term human DEC-205 presumably encompasses full length human DEC-205, as well as undescribed mutants and alleles of human DEC-205.

B. Appellants' Response

1.   Each Independent Claim Requires Separate Consideration

Appellants respectfully disagree with the Examiner's rejection. As a preliminary matter, the scope of claims 6-9 and 13-21 varies and, as such, the assertions made by the Examiner are not equally applicable to all of these claims.

Specifically, contrary to the Examiner's opinion that the claimed antibody conjugates do not bind to any specific epitope of human DEC-205, claims 13-17 are drawn to antibody conjugates which do, indeed, bind to a particular epitope of human DEC-205, namely the C-terminal sequence (SEQ ID NO: 7).

Similarly, that the present specification teaches a partial human DEC-205 sequence is also irrelevant with respect to claims 18-21, since these claims are drawn to methods employing antibody conjugates that bind to *full length murine DEC-205*

protein (SEQ ID NO: 10). Thus, the Examiner's statement that the antibody conjugates of claims 18-21 bind to "undisclosed amino acids of DEC 205" is incorrect. Indeed, the full length sequence of murine DEC 205 is explicitly provided in the present application as SEQ ID NO: 10. Moreover, while the antibody conjugates of claims 18-21 also cross-react with human DEC-205, the epitopes of human DEC-205 that the conjugates bind to are thus, by definition, shared with (i.e., cross-reactive with) murine DEC-205. As such, the sequence of these epitopes is provided as part of the full length murine DEC-205 sequence recited in the claims (SEQ ID NO:10).

For at least the reasons above, the reasons provided by the Examiner for rejecting claims 13-17 and 18-21 as lacking written description under 35 U.S.C. §112, first paragraph, do not apply or support the rejection.

Finally, with respect to claims 6- 9, drawn to methods which employ antibody conjugates that bind to human DEC-205 protein comprising the partial amino acid sequence of SEQ ID NO:7, Appellants respectfully submit that while Appellants' specification does not recite the full length human DEC-205 sequence, or the sequence of each and every variant of human DEC-205, this does not *de facto* mean that the pending claims fail to comply with the written description requirement. Importantly, it is well-established that the written description standard is not a bright line test, but instead takes into consideration a number of different factors. As discussed in detail below, Appellants' disclosure of the partial human DEC-205 sequence and the full length murine DEC-205 sequence, in combination with knowledge available in the art, were sufficient to demonstrate to one of ordinary skill that they had full possession of the complete human DEC-205 protein, and antibody conjugates against the protein, at the time the present application was filed.

2.   The Descriptive Text Needed to Satisfy the Written Description Standard Must be Considered in Relation to the Scientific Knowledge in Existence at the time of the Invention, the Skill in the Art, and Correlation of a Disclosed Function to a Known Structure

The mere fact that Appellants' specification does not recite the full length human DEC-205 sequence does not alone mean that any of the claims on appeal fail to comply with the written description requirement.

Moreover, Appellants respectfully disagree with the Examiner's assertion that the decision in *Capon v. Eshhar* (418 F.3d 1349, 1357 (Fed. Cir. 2005)) "is not relevant to the claims under consideration." While the claims on appeal may differ from the claims on appeal in *Capon v. Eshhar*, the Court took considerable effort to lay out the underlying framework for determining written description in other cases moving forward, and to clarify that written description, like enablement, must be determined on a case by case basis. Specifically, the standard for meeting the written description requirement and showing possession of the claimed invention, as articulated by *Capon v. Eshhar*, differs for every patent specification depending upon a number of factors, including the scientific knowledge in existence at the time of the invention, the skill in the art, the predictability of the claimed subject matter, and correlation of a described function to a known structure. Again, Appellants do not argue that the claims at issue in *Capon v. Eshhar* were the same as in the present case, rather that the written description standard articulated by the Court, when applied in the present case, is fully satisfied.

Specifically, as discussed further below, the maturity of the science and skill in the art at the filing date of the present invention were such that one of ordinary skill could predictably obtain full-length proteins, such as DEC-205, based on partial sequences, as well as predictably obtain antibodies against the full-length protein (or any region or variants of the protein). As such, Appellants teachings in the specification, combined with the knowledge available in the art, demonstrate that Appellants were in full possession of the presently claimed invention at the time of filing.

3.   Isolation and Cloning of Proteins, and Generation of Antibodies Were Highly Mature Technologies at the Time of the Present Invention

Indeed, at the filing date of the present application (*i.e.*, in 1995), technologies for isolating, characterizing and cloning proteins were highly developed, as were technologies for generating antibodies against such proteins. For example, several well known techniques were available for cloning proteins, including human DEC-205, based on a given partial amino acid sequence of the protein (see, for example, page 20, line 30 through page 21, lines 1-19; as well as page 25, lines 25-31 through page 31, lines 1-16 of the parent application, USSN 09/586,704). Additionally, techniques for

expressing cloned proteins (see, for example, page 31, lines 18-31 through page 35, lines 1-30 of the parent application, USSN 09/586,704) and for generating antibodies against the proteins were equally well known (see, for example, page 42, lines 23-31 through page 45, lines 1-19, and particularly page 42, lines 28-31 in the parent application, USSN 09/586,704). Once armed with a partial amino acid (*i.e.,* a peptide derived from a given protein), it was also well within the skill of the art to use these techniques to generate antibodies against such peptides and to isolate the full-length protein from its natural source.

Appellants specifically illustrated this in relation to mouse DEC-205. In particular, Appellants successfully isolated and characterize full-length mouse DEC-205 from whole murine thymus using mAb NLDC-145, an anti-mouse DEC-205 antibody (see page 63 of the parent application, USSN 09/586,704). Additionally, Appellants successfully raised antibodies against N-terminal peptides from mouse DEC-205 protein (see, for example, page 62, lines 26-32 and page 63, lines 1-15 of the parent application, USSN 09/586,704). This provides *clear evidence* that the partial human DEC-205 sequence described in the present disclosure put Appellants in possession of the complete DEC-205 protein and antibodies against the protein.

Additionally, in the present application, Appellants teach a partial (C-terminal) sequence (SEQ ID NO.:7) of human DEC-205 protein. Appellants further teach the highly homologous full-length sequence of mouse DEC-205 protein (SEQ ID NO.:10), along with an in-depth characterization of this protein (including its ability to deliver antigen to an active antigen processing compartment of dendritic cells). Appellants also describe well-known techniques for cloning proteins (including human DEC-205) based on a given partial amino acid sequence of the protein, expressing cloned proteins and generating antibodies against the proteins. Based on these teachings, it was well within the skill of the art to have generated anti-DEC-205 antibodies. It was also well within the skill in the art to have generated full-length human DEC-205 protein, as well as variants of the human DEC-205 protein.

In fact, as evidenced by the Declaration by Dr. Michel Nussensweig (Appendix B), the cloning techniques and techniques for generating antibodies described in the specification were ultimately successfully used to clone and isolate human DEC-205 and to produce antibodies against full-length human DEC-205. This provides clear evidence that Appellants were in fact indeed in possession of the claimed invention

based on the descriptive text provided within the four corners of Appellants' originally filed disclosure.

4. The Structure and Function of Human DEC-205 Correlates to the Structure and Function of Mouse DEC-205 Protein

Finally, the Written Description requirement may be satisfied if the disclosed function of the claimed invention sufficiently correlates to a particular, known structure. In the present case, the structure and function of human DEC-205 clearly correlates to that of mouse DEC-205, the characteristics of which (including full-length sequence) are described in detail in the present disclosure. Accordingly, the fact that Appellants provide an in-depth characterization of mouse DEC-205, including its full-length sequence, which correlates to human DEC-205, provides further basis for fully meeting the Written Description requirement.

In sum, the teachings set forth in Appellants' specification, in combination with the high level of skill and knowledge in the art at the time of the invention, and the proven predictability of the technologies involved in the invention, clearly satisfies the standard for Written Description according to the guidelines articulated by the CAFC in *Capon v. Eshhar* (CAFC 2005), and demonstrates possession of the claimed invention.

C. Summary of Examiner's Rejection of Claims 6-9 and 13-17 Under 35 U.S.C. § 112, First Paragraph, as Failing to Comply with the Written Description Requirement

The Examiner has rejected claims 6-9 and 13-17 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. In particular, the Examiner asserts that there is no support in the specification for a human DEC-205 protein comprising an amino acid sequence as set forth in SEQ ID NO.:7. The Examiner further asserts that, although the specification teaches that SEQ ID NO.:7 is a peptide derived from DEC-205, there is no support for a DEC-205 protein comprising the peptide wherein the molecule could have any amino acids in association with the aforementioned sequences recited in the claim.

D. Appellants' Response

As an initial point, it is unclear to Appellants, based on the Examiner's comments, what the distinction is between the former 35 U.S.C. § 112, first paragraph, rejection of claims 6-9 and 13-21, and the present § 112, first paragraph, rejection of claims 6-9 and 13-17. Indeed, both rejections appear to be based on the same premise, *i.e*, that the claims lack written description because the specification teaches a partial human DEC 205 sequence. Appellants note, however, that the former rejection has been applied to claims 6-9 and 13-21, whereas the present rejection has been applied only to claims 6-9 and 13-17.

Accordingly, with respect to claims 13-17, Appellants again respectfully note that these claims are drawn to methods that employ antibody conjugates defined as binding to a *particular* epitope on human DEC 205, the sequence of which is explicitly taught in the application (SEQ ID NO:7). Therefore, the Examiner's assertion that the specification fails to provide support for a human DEC 205 protein comprising the partial sequence of SEQ ID NO:7 does not provide a basis for rejecting claims 13-17 for lack of written description.

Moreover, for the many reasons discussed above in Section B, Appellants respectfully submit that the specification does indeed provide full support for a human DEC 205 protein comprising SEQ ID NO:7, as recited in claims 6-9. Again, the mere fact that the disclosure teaches partial sequences for human DEC 205 does not alone mean that the claims covering antibody conjugates which bind to human DEC 205 comprising such sequences lack written description. Whether claims 6-9 comply with § 112, first paragraph, depends on a variety of factors, as discussed above in relation to the previous rejection (Section B). When applied in the present case, given the teachings in Appellants' specification, in combination with the skill and knowledge available in the art at the time the present application was filed, clearly demonstrate that Appellants possessed the complete human DEC-205 protein recited in claims 6-9.

As previously discussed in detail, Appellants teach the partial C-terminal sequence of human DEC-205 (SEQ ID NO: 7). Based on this partial amino acid sequence, it was well within the skill of the art to have used known techniques to generate antibodies against this peptide, and to have predictably isolated the full-length protein or variants from its natural source. In fact, the maturity of the science and skill in the art at the time of the present invention were such that those of ordinary skill in

the art were routinely obtaining full-length proteins based on partial sequences, as well as predictably obtaining antibodies against such full-length proteins . This is specifically attested to in the Declaration submitted by Declaration by Dr. Michel Nussensweig (Appendix B). Further, the fact that Appellants provide an in-depth characterization of mouse DEC-205, including its full-length sequence, which correlates to human DEC-205, provides further basis for fully meeting the Written Description requirement.

In sum, for at least the foregoing reasons, claims 6-9 and 13-21 fully comply with 35 U.S.C. § 112, first paragraph.

VIII.   CONCLUSION

Appellants submit that claims 6-9 and 13-21 comply with the written description requirement under 35 U.S.C. § 112, first paragraph, and respectfully request that the Board reverse the rejection of claims 6- 9 and 13-21 for the reasons set forth above.

Dated: September 28, 2007

Respectfully submitted,

By _____
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CLAIMS APPENDIX A

6.   (Previously Presented) A method for enhancing the development of tolerance to a preselected antigen for which tolerance is desired, in a mammal comprising exposing ex vivo or in vivo dendritic cells from said mammal to a conjugate comprising said preselected antigen covalently bound to an anti-human DEC-205 antibody or an anti-murine DEC-205 antibody that binds to human DEC-205 under conditions that promote dendritic cell quiescence, said human DEC-205 protein comprising an amino acid sequence as set forth in SEQ ID NO: 7, and wherein said preselected antigen is selected from the group consisting of allergens, autoantigens and antigens participating in allograft rejection.

7.   (Original) The method of claim 6 wherein said preselected antigen is a peptide antigen or a protein antigen.

8.   (Original) The method of claim 7 wherein said peptide or protein is conjugated to said antibody to DEC-205 by means of a cross-linking agent.

9.   (Original) The method of claim 7 wherein a light chain or a heavy chain of said antibody to DEC-205, and said peptide antigen or protein antigen, are present on a single polypeptide chain.

13.   (Previously Presented) A method for enhancing the development of tolerance to a preselected antigen for which tolerance is desired in a mammal, comprising exposing ex vivo or in vivo dendritic cells from said mammal to a conjugate comprising said preselected antigen covalently bound to an anti-human DEC-205 antibody, wherein the antibody is reactive with an amino acid sequence as set forth in SEQ ID NO: 7, under conditions that promote dendritic cell quiescence, wherein said preselected antigen is selected from the group consisting of allergens, autoantigens and antigens participating in allograft rejection.

14.   (Previously Presented) A method for enhancing the development of tolerance to a preselected antigen for which tolerance is desired, in a mammal comprising exposing

ex vivo or in vivo dendritic cells from said mammal to a conjugate comprising said preselected antigen covalently bound to an anti-murine DEC-205 antibody, wherein the antibody is reactive with an amino acid sequence as set forth in SEQ ID NO: 7, under conditions that promote dendritic cell quiescence, and wherein said preselected antigen is selected from the group consisting of allergens, autoantigens and antigens participating in allograft rejection.

15.   (Previously Presented) The method of either one of claims 13 or 14, wherein said preselected antigen is a peptide antigen or a protein antigen.

16.   (Previously Presented) The method of either one of claims 13 or 14, wherein said peptide or protein antigen is conjugated to said antibody to DEC-205 by means of a cross-linking agent.

17.   (Previously Presented) The method of either one of claims 13 or 14, wherein a light chain or a heavy chain of said antibody to DEC-205, and said peptide antigen or protein antigen, are present on a single polypeptide chain.

18.   (Previously Presented) A method for enhancing the development of tolerance to a preselected antigen in a mammal, the method comprising exposing ex vivo or in vivo dendritic cells from the mammal to a conjugate comprising the preselected antigen bound to an anti-mouse DEC-205 antibody that cross reacts with human DEC-205 under conditions that promote dendritic cell quiescence, wherein the mouse DEC-205 protein comprises the amino acid sequence of SEQ ID NO: 10.

19.   (Previously Presented) The method of claim 18, wherein the preselected antigen is selected from the group consisting of allergens, autoantigens and antigens participating in allograft rejection.

20.   (Previously Presented) The method of claim 19, wherein the preselected antigen is bound to the antibody to DEC-205 by means of a cross-linking agent.

21.   (Previously Presented) The method of claim 18, wherein a light chain or a heavy chain of the antibody to DEC-205, and the preselected antigen, are present on a single polypeptide chain.

EVIDENCE APPENDIX

Appendix B is a copy of the Declaration by Dr. Michel Nussensweig, which was entered by the Examiner in conjunction with the Amendment and Response filed by Appellants on January 4, 2005.

Appendix C is a copy of Guo *et al.* (Hum Immunol. 2000 Aug; 61(8):729-38), which was referenced in the Declaration by Dr. Michel Nussensweig and cited in an Information Disclosure statement (dated December 27, 2005) that was considered and initialed by the Examiner on March 19, 2006.

RELATED PROCEEDINGS APPENDIX

Please note that an Appeal Brief has been filed in the parent application, U.S.S.N.: 09/586,704.
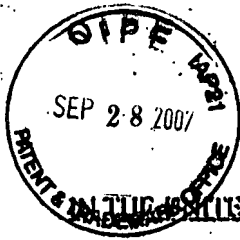
600-1-081CONCIP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Steinman et al.   EXAMINER: Schwadron, Ronald B.

SERIAL. NO.: 09/925,284   ART UNIT: 1644

FILED: August 9, 2001

FOR: ENHANCED ANTIGEN DELIVERY AND MODULATION OF THE
IMMUNE RESPONSE THEREFROM

DECLARATION UNDER 37 C.F.R. 1.132

COMMISSIONER FOR PATENTS
P.O. BOX 1450
ALEXANDRIA, VIRGINIA 22313-1450

SIR:

I, MICHEL NUSSENZWEIG, hereby declare and state that:

1. I am a Howard Hughes Investigator, Sherman Fairchild Professor and Senior Physician at Rockefeller University, having received my Ph.D. degree from the Rockefeller University in 1981 and my M.D. degree from New York University in 1982. I received postdoctoral medical and scientific training at Harvard University. My full curriculum vitae is attached hereto as Exhibit A.

2. My principal area of research is in Immunology and among other positions I serve as reviewer in numerous funding agencies of many countries, including the National Institute of Health, March of Dimes, Dana Foundation. I also have served as reviewer for numerous scientific journals, and I am the Editor of the Journal of Experimental Medicine and the Journal of Immunologic Methods.

3. In the course of my activities, I have been listed as inventor on several patent applications, including the one noted above entitled "ENHANCED ANTIGEN DELIVERY AND MODULATION OF THE IMMUNE RESPONSE THEREFROM", having U.S. Serial Number 09/925,284, which is a continuation-in-part of U.S. application Serial Number 09/586,704, filed on June 5, 2000, which is a continuation of U.S. Serial Number 08/381,528, filed on January 31, 1995, now abandoned.

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4. I have reviewed the disclosure of the present application, with particular emphasis on the support in the application as filed for the preparation and generation of antibodies against human and mouse DEC-205 proteins.

5. The present application claims a method of enhancing the development of tolerance to a pre-selected antigen for which tolerance is desired in a mammal comprising exposing ex vivo or in vivo dendritic cells from said mammal to a conjugate comprising said pre-selected antigen covalently bound to an anti-human DEC-205 antibody or an anti-murine DEC-205 antibody that binds to human DEC-205 under conditions that promote dendritic cell quiescence. More particularly, the pre-selected antigen is selected from the group consisting of allergens, autoantigens and antigens participating in allograft rejection. The human DEC-205 protein has a carboxy terminal and amino terminal amino acid sequence as disclosed in the parent application, U.S. Serial Number 09/586,704, filed on June 5, 2000, as SEQ ID NOs.: 1 and 2, respectively. Furthermore, as noted in the parent application USSN 09/586,704, the first 19 amino acid residues of the amino terminal human DEC-205 protein (designated as SEQ ID NO: 13 in the parent application) were used to generate antibodies that reacted with human DEC-205. The sequences from the parent application have now been included in the sequence listing for the present application and are designated as SEQ ID NO: 7 for the carboxy terminal, SEQ ID NO: 8 for the amino terminal, and SEQ ID NO: 9 for the first 19 amino acids of the amino terminal used for antibody generation.

6. The subject matter of the present application was based on work performed in my laboratory, whereby the human DEC-205 molecule was cloned and expressed (Guo, M., Gong, S., Marie, S., Misulovin, Z., Pack, M., Mahnke, K., Nussenzweig, M.C. & Steinman, R.; (2000), A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells, *Human Immunology* 61:729-738). Anti-human DEC-205 antibodies were then prepared by immunizing animals with the first 19 amino acid residues from the N terminal fragment of the cloned human DEC-205 protein.

7. To summarize briefly, the cloning of human DEC-205 was done through use of a cDNA fragment of the 3' portion of mouse DEC-205. This was used to screen a human lymphocyte and thymus cDNA library using standard procedures known to those skilled in the art. In particular, the cDNA fragment of mouse DEC-205 was used to screen a human lymphocyte matchmaker cDNA library (EBV-transformed human peripheral blood B lymphocytes) and a human thymus 5'-stretch plus cDNA library in a Ogt10 vector (Clontech Laboratories, Palo Alto, CA, USA). Positive clones were identified by DNA sequencing on

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both strands using Sequenase (United State Biochemical, Cleveland, OH, USA), or the dye determinator kit (PE Applied Biosystems, Foster City, CA, USA) and automated sequencing (Appplied Biosystems model 371). The human cDNAs were expressed in pEF-BOS modified to carry a 3' human Fc fragment that was in frame with the insert. DEC-205 leader, CR domain, and FnII domains were amplified from plasmids by PCR using 5' MG31 primers and 3' MG35 primers. The 5' – primer contains a SpeI site, while the 3' – primer contains a NotI site and codes for PRR at the junction point of DEC-205 and the Fc tag. The human DEC-205 Fc fusion protein was produced by transiently transfecting 293 cells using calcium phosphate mediated gene transfer. The fusion protein was purified on protein A sepharose and was then used to inject mice. Following several booster injections, the serum was tested for antibodies that reacted with the CR-FnII domain of the human DEC-205 molecule using Western blot procedures. Afterwards, the spleens were harvested from those animals showing a positive reaction and were fused with SP2/0 cells. The supernatants were screened by ELISA, dot blot, thymus tissue staining and FACS analysis. Cell clones that secreted anti-human DEC-205 antibodies were further subcloned and expanded.

8. The present application tonches methods for inducing tolerance by conjugating an antigen to a DEC-205 antibody for targeting to the DEC-205 receptor on specific cells, such as dendritic cells, under conditions that promote dendritic cell quiescence. The antibodies that react with the DEC-205 proteins, in particular, the anti-human DEC-205 antibodies, were prepared using the first 19 amino acid residues from the amino terminal end of the cloned human DEC-205 protein, as described in the parent application U.S. Serial Number 09/586,704, and further attested to in this declaration. Thus, it is my belief that the disclosure of the present application provides sufficient written description for a person skilled in the art to prepare such antibodies that react with human DEC-205 protein as presently claimed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the U.S. Code, Section 1001, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Dated: 1/3/0.5

Michel Nussenzweig, M.D., Ph.D.

EXHIBIT A

CURRICULUM VITAE

Name: Michel C. Nussenzweig

Date of Birth: February 10, 1955

Education:
1975   B.A. - New York University College of Arts and Sciences
1981   Ph.D. - The Rockefeller University
1982   M.D. - New York University School of Medicine

Clinical Training:
1982-1985   Intern & Resident, Internal Medicine
                   Massachusetts General Hospital
1984-1985   Clinical Fellow, Infectious Diseases
                   Massachusetts General Hospital

Postdoctoral Training:
1986-1989   Harvard Medical School, Department of Genetics

Professional Appointments
1990-1996   Assistant & Associate Professor, The Rockefeller University
1990-1999   Assistant & Associate Investigator, Howard Hughes Medical Institute
1996-present   Professor & Senior Physician, The Rockefeller University
1999-present   Investigator, Howard Hughes Medical Institute
2000-present   Sherman Fairchild Professor of Immunology, The Rockefeller
Univ.

Honors & Awards
Summa Cum Laude, New York University College of Arts and Sciences - 1975; Phi Beta Kappa,
New York University College of Arts and Sciences - 1975; Alpha Omega Alpha, New York
University Medical School - 1982; Bertram M. Gresner Memorial Research Award, New York
University School of Medicine – 1982; Elected Member American Society of Clinical
Investigators - 1997, Solomon A. Berson Award for Basic Science - 2002

Teaching:
Immunology, Course Organizer

Institutional:

Chair, Transgenic Facility Coordinating Committee
Chair, Animal Care and Use Committee

Chair, Hospital Seminar Committee
Member, Immunology Search Committee
Member, Institutional Review Board for Biohazards, Radioisotopes, Toxic Chemicals, and
  Carcinogens
Member Hospital GCRC Scientific Advisory Committee
Elected Senior Faculty Representative Academic Council
Member, Virology Search Committee

National
Arthritis Foundation Molecular Immunology study section 1993-1996
NIH Immunobiology Study Section Ad Hoc reviewer 1998, and 1999
NIH ALY Study Section Ad Hoc Reviewer, 1999
NIH NIAID Council Ad Hoc 1998
Organizer Keystone Symposium on Dendritic Cells 1998
Organizer Keystone Symposium on B Cells 1999
March of Dimes Review Committee 1999-
External Reviewer LMGD NICHD 2000
Damon Runyon Cancer Research Fund Review Committee 2000-2002
American Association of Immunologists Program Committee 2000-
NIH ALY Study Section Member 2001-
Organizer Keystone Symposium on B Cell Biology 2003

Editorial:
1996-Present   Editor, The Journal of Experimental Medicine
1999-Present   Editor, The Journal of Immunological Methods
2000-Present   Transmitting Editor, International Immunology
2002-Present   Advisory Editor, Nature Reviews Immunology

Consultant:
Abgenix, Fremont, CA
Zycos, Lexington MA

Professional Memberships:
American Association of Immunologists
American Medical Association
The New York Academy of Sciences
Kunkel Society
Harvey Society

Publications:

1. Steinman, R.M., Witmer, M.D., **Nussenzweig, M.C.,** Chen, L.L. & Cohn, Z.A. Dendritic Cells: an important new cell type in the mixed leukocyte reaction. In: Kaplan JG, ed. Proc. of the 13th International Leukocyte Culture conference. *Elsevier/North Holland Publ.* Co. 273. (1979)

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A Monoclonal Antibody to the DEC-205 Endocytosis Receptor on Human Dendritic Cells

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ABSTRACT: DEC-205 is a multilectin receptor for adsorptive endocytosis, expressed in mouse dendritic cells (DC) and some epithelia. DEC-205 is homologous to the macrophage mannose receptor (MMR). A cDNA for murine DEC-205 was used to identify 3 overlapping human DEC-205 clones from a lymphocyte library. The human homologue is a transmembrane protein of 1722 aminoacids with 10 externally disposed C-type lectin domains having 77% identity to the mouse counterpart. The NH_2 terminal cysteine-rich and fibronectin type II domains were expressed and used to immunize mice. A hybridoma, MG38, which specifically recognized the immunogen was obtained from a DEC-205 knockout mouse. The antibody precipitated a 205 kD protein from metabolically labeled, monocyte-derived DCs. MG38 labeled mature monocyte-derived DCs but showed weak or no labeling of other peripheral blood mononuclear cells. In tissue sections, MG38 identified DEC-205 on thymic cortical epithelium and DCs in the thymic medulla and tonsillar T cell areas. In contrast, an anti-MMR antibody stained DEC-205 negative, macrophages in the thymus cortex, the trabeculae of the thymus and tonsil, as well as efferent lymphatics in the tonsil. Therefore, the MG38 anti-DEC-205 antibody is useful for identifying DCs and reveals clear differences in sites where MMR and DEC-205 are expressed in lymphoid tissues. Human Immunology 61, 729–738 (2000). © American Society for Histocompatibility and Immunogenetics, 2000. Published by Elsevier Science Inc.

KEYWORDS: dendritic cells; antigen receptor; C-type lectin; DEC-205; monoclonal antibody

ABBREVIATIONS

MMR   macrophage mannose receptor

DC   dendritic cell

INTRODUCTION

Several receptors for adsorptive endocytosis and antigen presentation have now been identified on dendritic cells (DCs). These include Fcγ [1] and Fcε [2] receptors and two multilectin receptors, the macrophage mannose receptor (MMR) [3] and DEC-205 [1]. The carbohydrate recognition domains of the MMR show specificity for mannose [3], and the MMR internalizes and presents mannosylated proteins [3, 4]. The ligands for DEC-205 are not yet known, but antibodies to DEC-205 are presented efficiently to Ig-reactive T cells [1]. Both the MMR and DEC-205 contain homologous amino terminal cysteine rich (CR) and fibronectin II domains followed by 8 and 10 contiguous C-type lectin domains respectively. Both molecules contain a single transmembrane domain and a cytosolic region with a coated pit localization sequence for adsorptive uptake.

DEC-205 is expressed by many different sources of mouse DCs [5–7], according to data with a rat-anti-mouse monoclonal antibody, originally termed NLDC-145 [5]. This monoclonal was used to clone DEC-205

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and provide the first information on its function [1]. A monoclonal to mouse MMR has yet to be reported. In contrast, the human MMR is a potent immunogen in mice, and the corresponding monoclonals have revealed abundant expression of the MMR on monocyte-derived DCs [3]. No antibody to human DEC-205 is available, although HuDEC-205 has been cloned independently in our laboratory and by Kato et al. [8].

Here, we set out to prepare monoclonal antibodies to sequences from the predicted protein but only succeeded when we immunized DEC-205 knockout mice. We describe a monoclonal MG38 to huDEC-205. Expression of human DEC-205 is prominent in DCs rather than other cells in human blood. Using monoclonals to MMR and DEC-205, we find that these receptors are expressed in different sites in tissue sections of thymus and tonsil.

MATERIALS AND METHODS

Human DEC-205 cDNA

A cDNA fragment of the 3' portion of murine DEC-205 was used to screen a human lymphocyte matchmaker cDNA library (EBV-transformed human peripheral blood B lymphocytes) and a human thymus 5'-stretch plus cDNA library in a Ogt10 vector (Clontech Laboratories, Palo Alto, CA, USA). Positive clones were characterized by DNA sequencing on both strands using Sequenase (United State Biochemical, Cleveland, OH, USA), or the dye terminator kit (PE Applied Biosystems, Foster City, CA, USA) and automated sequencing (Applied Biosystems model 371).

Expression of CR-FnII Domains of hDEC-205 in a Mammalian 293T Cell Expression System

cDNAs were expressed in pEF-BOS [9] modified to carry a 3' human Fc fragment that was in frame with the insert. DEC-205 leader, CR domain, and FnII domains were amplified from plasmids by PCR using 5'-primers MG31: 5'-CGGGATCCACTAGTCGCGTGCGCCCGAGG-3', and 3'-primer MG35: 5'-CTTAAAGCCTGAAAACG GTCCGCGCCGGGCGGCCGCATTCTTAT-3'. The 5'-primer contains a SpeI site, while the 3'-primer contains a Not I site and codes for PRR at the junction point of DEC-205 and the Fc tag (Fig. 1A). The hDEC-205-Fc fusion protein was produced by transiently transfecting 293T cells using calcium phosphate-mediated gene transfer. Medium containing the secreted recombinant protein was harvested after 10–12 days, and the recombinant protein was recovered by protein A chromatography [10].

Proteolysis and Antigen Purification

The CR-FnII portion of the fusion protein was separated from the Fc tag by papain digestion. Fusion protein
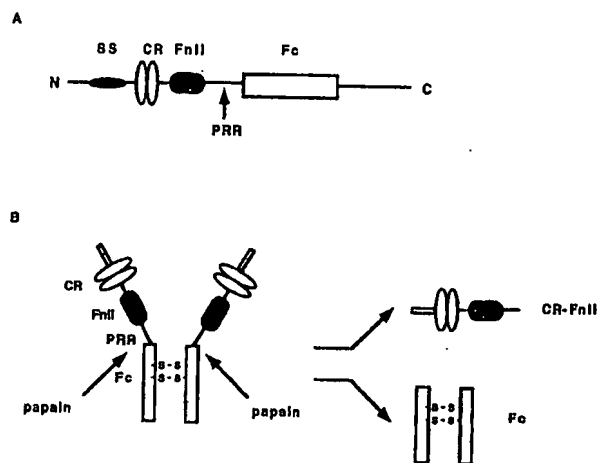

FIGURE 1 (A) Schematic of the CR-FnII-Fc fusion protein expression construct. SS: signal sequence; CR: cysteine-rich domain; FnII: fibronectin type II domain; Fc: human IgG Fc domain; PRR: three amino acids, proline-arginine-arginine, inserted at the junction point of the DEC domain and Fc domain. (B) Schematic of fragments produced by papain digestion. The secreted fusion protein forms dimers through S-S bonds in the Fc domain. Papain cleaves at the end of the Fc domain close to the junction point.

bound to Protein A Sepharose was digested with papain (Sigma Chemical Co., St. Louis, MO, USA) at a ratio of enzyme:protein of 1:100 in 1X PBS containing 10 mM EDTA and 10 mM cysteine for 30 min at 37°C (Fig. 1B). The reaction was terminated by adding iodoacetamide to a final concentration of 30 mM. Protein A beads and the bound Fc fragments were removed by centrifugation.

Immunization of DEC-205 Knockout Mice and Hybridoma Production

Purified CR-FnII protein in CFA (prime) or IFA (boost) were injected intraperitonally four times into DEC-205 knock out (DEC-205$^{-/-}$) mice at 50 μg per inoculation. The DEC-205$^{-/-}$ mice were produced by deleting the DEC-205 promoter and first coding exon in ES cells as previously performed for Igβ [11]. Initial immunophenotyping showed apparently normal B and T cell development in these mice, as well as T cell dependent immune responses. Sera were harvested from immunized mice and tested for the presence of anti-CR-FnII by western blot. Mice producing antibodies were reboosted with CR-FnII protein in PBS, their spleens were harvested 4 days later, and the splenocytes were fused with SP2/0 cells [10]. Supernatants were screened by ELISA, dot blot, thymus tissue section staining, and FACS analysis.

ELISA

Plates were coated with purified CR-FnII protein at a concentration of 2 μg/ml at 37°C for 2 hrs and blocked

30 min at room temperature with blocking buffer (0.25% BSA in PBS). Fifty μl of supernatant from the growing fusion well was added and incubated at room temperature for 1 h, then washed. HRP-conjugated secondary antibody (1:5,000 dilution in blocking buffer) was added and incubated for 45–60 min at room temperature, then washed. Samples were developed using HRP substrate reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Dot Blot

Ten to one hundred nanograms of antigen was loaded directly on the nitrocellulose paper. The nitrocellulose paper was blocked for 30 min at room temperature with 5% dry milk in wash buffer (PBS with 0.05% Tween 20), then incubated with hybridoma supernatants for 60 min at room temperature, and washed with washing buffer. HRP- conjugated-goat anti-mouse antibody (1: 10,000 dilution in washing buffer) was added and incubated for 45 min at room temperature, then washed. The blots were developed with ECL detection reagent (NEN Life Science Products, Inc., Boston, MA, USA).

Preparation of Cells

PBMCs, monocytes, T cells, DCs, and macrophages were prepared from the blood of normal donors as described [12]. After isolation by sedimentation in Ficoll-Hypaque, PBMC were cultured in RPMI-5% human serum for 1 h. Floating cells were removed, and adherent cells were incubated 6 days in RPMI-5% human serum with GM-CSF (100 IU/ml, Leukine, Immunex) and IL-4 (1000 U/ml, Genzyme). Cells were fed on days 2, 4, and 6 with the same medium. Nonattached cells were harvested on day 6 as immature DCs, and to make mature DCs, the cells were cultured 3 more days in the above medium, cytokines and LPS. Macrophages were also obtained from PBMC by culture in parallel but in the absence of cytokines.

Immunohistochemistry

Frozen sections were cut at 10 μ from surgical specimens of human tonsil and thymus, generously provided by Dr. W.A. Muller (Cornell University Medical College, New York, NY, USA). Sections were cut onto glass slides (Carlson, Peotone, IL, USA), air dried, fixed in acetone and stained as follows. Biotinylated monoclonal antibodies, MG38 anti-DEC-205 and 3.29 anti-MMR [3] (a gift of Dr. A. Lanzavecchia, Basel), were applied at 20 and 5 μg/ml, respectively. Alkaline phosphatase ABC (Vector) was applied for 30 min and the enzyme reaction developed using a BCIP/NBT substrate kit (Vector, Burlingame, CA, USA) according to manufacturer's instructions. Mouse serum (10%) was applied for 15 min, followed by leu 2 anti-CD8 hybridoma culture superna-

tant overnight and POX-anti mouse Ig (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. Stable DAB (Research Genetics, Huntsville, AL, USA) was used for 6 min to develop the HRP reaction. The slides were mounted in PBS/glycerol. Alternatively, for two color immunofluorescence, thymic sections were labeled with the following sequence: 3.29 MMR at 2 μg/ml followed by FITC-goat anti-human IgG1, then MG38 anti-DEC-205 at 2 μg/ml followed by biotin goat anti-human IgG2b and Cy3 Streptavidin (all secondary antibodies from PharMingen). Stacks of 0.5 μ optical sections were taken by an Olympus epifluorescence microscope equipped with a motorized stage, a cooled CCD camera (Hamamatsu, Japan), and Metamorph software (Universal Imaging). Images were deconvoluted by applying a nearest neighbor algorithm provided by Metamorph.

Flow Cytometry

Fresh or permeabilized cells were incubated with hybridoma culture supernatant for 30 min on ice, washed with PBS-1% fetal calf serum, incubated with secondary antibody for 30 min on ice, then washed. Immunoglobulins were used at 1 μg/ml and mAb-containing supernatant was used at 1:1 dilution. The secondary antibody was fluorescein isothiocyanate (FITC)-goat-anti-mouse immunoglobulin (1:200 dilution, Jackson). PE-CD14 (Becton Dickinson, Mansfield, MA, USA), PE-HLA-DR (Becton Dickinson), PE-CD83 (Immunotech) were used for double staining. The antibody to DC-LAMP [13] was provided by Drs. S. Lebecque and S. Saeland of the Laboratory for Immunology Research, Dardilly France. Stained cells were analyzed using FACScan flow cytometer (Becton Dickinson). For permeabilization, cells were fixed in 4% paraformaldehyde (Fisher Scientific Co., Pittsburgh, PA, USA) freshly made in PBS for 30 min on ice, followed by two washes with PBS. Fixed cells were permeabilized with 0.5% saponin (Sigma Chemical Co., St. Louis, MO, USA) in PBS with 1% FCS for 30 min on ice and washed twice using PBS with 1% FCS. To rule out Fc receptor-mediated binding of MG38 to DCs, we verified that the results in the FACS and tissue section studies were not altered by inclusion of 10% human serum in the staining reactions.

Cell Lysates and Metabolic Labeling

Monocytes, T cells, and DCs were harvested, counted, and washed three times with 1× PBS, then resuspended in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM tris-HCl). The following components were added to RIPA buffer before use: 1 mM DTT, 1 mM PMSF and 1× proteinase inhibitor cocktail (1 mg/ml leupeptin, 1 mg/ml antipain, 10 mg/ml benzamidine hydrochloride, 5 mg/ml aprotinin, 10 mg/ml soy bean trypsin Inhibitor, 1 mg/ml pepstatin). Samples

were rotated at 4°C for 30 min, and nuclei removed by spinning in a microfuge for 10 min. Total protein concentrations of the cell lysate was determined using 1X Bradford Assay buffer reagent (Bio-Rad Laboratories, Hercules, CA, USA). For metabolic labeling, cells were washed once with 1X PBS, then resuspended at 1 × 10⁷ cells per 100 mm plate in 10 ml labeling media (DMEM, without methionine, supplemented with 10% dialyzed fetal bovine serum and antibiotics; Gibco-BRL, Grand Island, NY, USA) and incubated at 37°C for 30 min to allow depletion of the intracellular pools of sulfur-containing amino acids. Cells were then labeled by adding 1 mCi of (³⁵S) methionine, for 6 h and lysates were prepared as described.

Immunoprecipitation (IP)

Lysates were pre-cleared by incubating with 100 μl of prewashed 50% protein A Sepharose in the cold for 30–60 min. 0.5–1 μg of each test antibody was then mixed with 50 μl of pre-cleared cell lysate and incubated on ice for 1 hr, and 30 μl of prewashed 50% protein A Sepharose was added for and additional hour. Beads were washed 3 times with cold RIPA buffer and analyzed by SDS-PAGE under reducing conditions.

RESULTS

Isolation of Human DEC-205

We previously reported the isolation of a cDNA clone encoding mouse DEC-205 [1]. By screening both human B cell and thymus libraries with probes derived from the mouse DEC-205 cDNA (see Materials and Methods), we obtained three overlapping cDNAs clones that when combined, encoded the complete human homologue of mouse DEC-205.

The predicted protein encoded by the hDEC-205 cDNAs is a type I transmembrane protein of 1722 amino acids with characteristic features of a group VI C-type lectin. Like mouse DEC-205 the human protein consists of an NH₂-terminal signal peptide followed by a cysteine-rich (CR) domain, a fibronectin type II (FnII) domain, ten carbohydrate recognition-like domains (CRDs), and a transmembrane domain, which is followed by a short cytoplasmic tail. Amino acid sequence alignment between the human and mouse cDNAs [1] reveals 77% identity, suggesting functional conservation. Our huDEC-205 sequence is identical to one independently isolated by Kato et al. [8].

Like mouse DEC-205, huDEC-205 is also homologous to other members of the multiple C-type lectin receptors, human macrophage mannose receptor (MMR, 22% identity), a membrane protein implicated in the endocytosis of glycoproteins bearing terminal mannose, fucose, N-acetylglucosamine, or glucose residues [4, 14];

and human phospholipases A2 (PLA₂) receptor (29% identity), which binds to PLA₂ that specifically catalyzes the hydrolysis of the 2-ester bond of 3-sn-phosphoglycerides [15, 16].

A Monoclonal Antibody Reactive with huDEC-205

Initial attempts to immunize mice with huDEC-205 failed to produce monoclonal antibodies. We reasoned that the failure might be due to the close conservation between human and mouse proteins. To overcome possible cross-tolerance we immunized DEC-knock-out mice. The fusion was initially screened with an ELISA assay against the purified CR-FnII fragment of the immunogen (see Materials and Methods). Reactive monoclonal antibodies were additionally tested by Western blot analysis. Clone MG38 specifically recognized and immunoprecipitated purified CR-FnII but not human Fc (Fig. 2A and B). These results demonstrate that MG38 specifically recognizes an epitope present in the CR-FnII region of human DEC-205. The final step of the screen was to verify MG38 staining of human thymic cortical epithelium, since mouse DEC-205 is abundant on this cell type [5, 6]. MG38 did not react with mouse DCs.

huDEC-205 Is Expressed by LPS Activated Monocyte-derived Dendritic Cells

To determine whether monocyte derived DCs expressed huDEC-205, we performed immunoprecipitation studies. Metabolically labeled monocyte-derived DCs yielded a single band migrating at a molecular weight that was greater than the 205 kDa seen with murine DEC-205 [7] (Fig. 3). A protein with the same molecular weight was also precipitated by rabbit polyclonal antibodies that were raised against mouse DEC-205 (unpublished results and [6, 7]). We presume that the higher apparent molecular weight of human DEC-205 reflects differences due to the primary sequence or postranslational modifications.

FACS Analysis of DEC-205 Expression

We used FACS analysis to additionally examine expression of huDEC-205 during DC development and maturation (Fig. 4). Immature DCs, mature DCs and macrophages were prepared from monocytes. Surface-bound as well as total DEC-205 was visualized in live and fixed-permeabilized cells, respectively. Other antibodies included a non-reactive IgG2b (termed Y-Ae; isotype-matched to MG38), CD21 (B cell marker), DC-LAMP (DC marker), and CD83 (DC marker). Double labeling was used to identify the DCs as CD14⁻ HLA-DR⁺⁺, and CD83⁺.

Only a small fraction of the immature DC preparation expressed high DEC-205 (Fig. 4A; <5% are DEC-205⁺ HLA-DR⁺, as indicated by the arrow). However, the
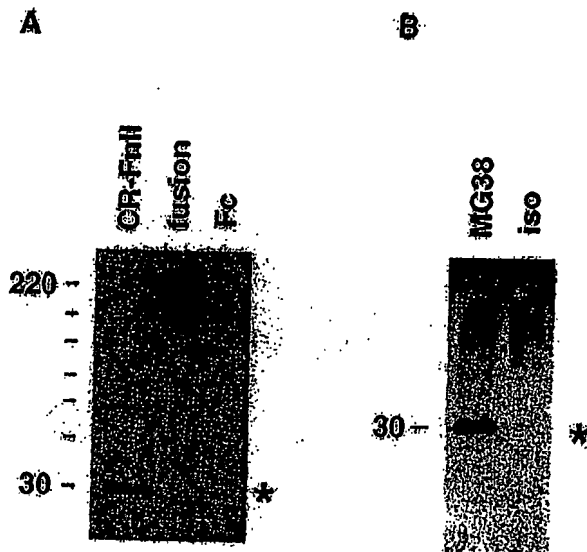
A

B

FIGURE 2 MG38 mAb reacts with CR-FnII domains of human DEC-205. (A) 50 ng of the CR-FnII and Fc regions of the fusion protein were separated by SDS-PAGE under non-reducing conditions, transferred to nitrocellulose paper and blotted with MG38. CR-FnII: papain digested and purified CR-FnII region of the fusion protein; fusion: the entire fusion protein; Fc: papain digested and purified Fc region of the fusion protein. MG38 specifically reacted with the CR-FnII protein (*) but not the Fc protein. 2 bands were seen in the fusion protein. The slow-migrating band was the dimer of full-length fusion protein; the fast-migrating band a heterodimer containing one full-length fusion protein and one Fc peptide, which resulted from cleavage of the fusion protein at the junction point during preparation. (B) Immunoprecipitation of CR-FnII by MG38. CR-FnII protein was incubated with MG38 or isotype-matched mAb as a control. The precipitated complexes were separated by SDS-PAGE under reducing conditions and visualized by blotting with rabbit-anti-CR-FnII antiserum. CR-FnII bands are indicated by an asterisk.

number of DEC-205+ cells was dramatically increased (>85%) in the mature DC population (Fig. 4A). Staining was increased considerably by permeabilizing the cells with saponin (Fig. 4A). Increased DEC-205 expression was correlated with higher levels of CD83 and DC-LAMP, both antigens being characteristic of mature DCs (Fig. 4B). In contrast, little or no staining was observed in other types of blood leukocytes present in PBMC (not shown). However, EBV transformed B-LCL did stain with MG38, which is not surprising given the fact that our human DEC-205 cDNA was obtained from a B-LCL library.

Differences in the Expression of MMR and DEC-205 in Sections of Lymphoid Tissue

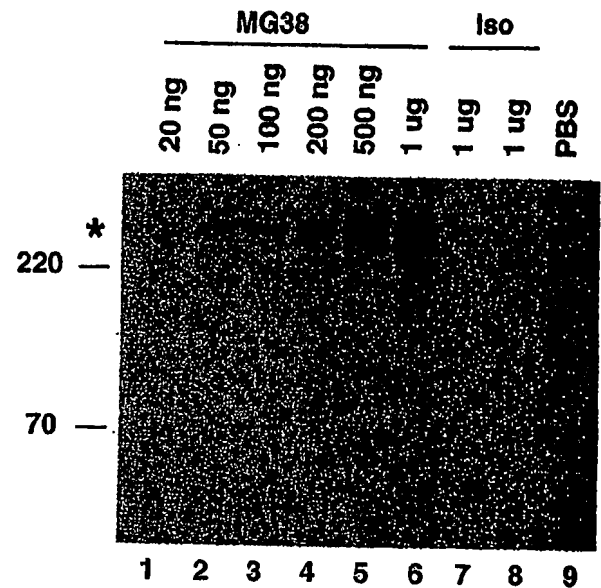Because monoclonals to human MMR had been prepared previously [3], we were in a position to use monoclonals

MG38   Iso

20 ng 50 ng 100 ng 200 ng 500 ng 1 ug 1 ug 1 ug PBS

1 2 3 4 5 6 7 8 9

FIGURE 3 Metabolic labeling of DEC-205. Lysates from metabolically-labeled mature DCs were incubated with MG38 in amounts ranging from 20 ng to 1 μg (lane 1 to lane 6). Increasing amounts of DEC-205 (*) were immunoprecipitated with increasing amounts of MG38, saturating at a level of 500 ng of MG38. The DEC-205 band was not obtained when using a control isotype-matched antibody (lane 7 and 8) or PBS (without Ab, lane 9).

to compare the in vivo expression of MMR with DEC-205. We studied sections of human tonsil and thymus. In tonsil, presumptive DCs in the T cell areas stained for DEC-205 (Fig. 5A, 5B). In contrast, the MMR was found on scattered presumptive macrophages in the septae; when found near the T cell area, the MMR was clearly present on vascular profiles, presumably efferent lymphatics (arrow, Fig. 5D). The latter originate in the T cell regions to carry tonsillar lymphocytes to the cervical and other lymph nodes in the neck (Fig. 5C, 5D). Neither DEC-205 nor MMR were found on blood vessels.

In thymus, DEC-205 stained the cortical epithelium (red stain in Fig. 6 left) and large dendritic profiles in the thymic medulla (red stain in Fig. 6 right). The latter medullary profiles double labeled for the DC-restricted marker DC-LAMP (not shown). In contrast, the MMR was found on scattered cells, presumably macrophages, in the thymic cortex and septae (green stain in Fig. 6 left) and on infrequent profiles in the medulla (green stain in Fig. 6 right). Two color immunofluoresence of thin 1 micron optical sections, examined by deconvolution microscopy, clearly showed that DEC-205 and MMR were expressed at different sites in the thymus. Therefore, these two homologous multilectins are expressed in dif-
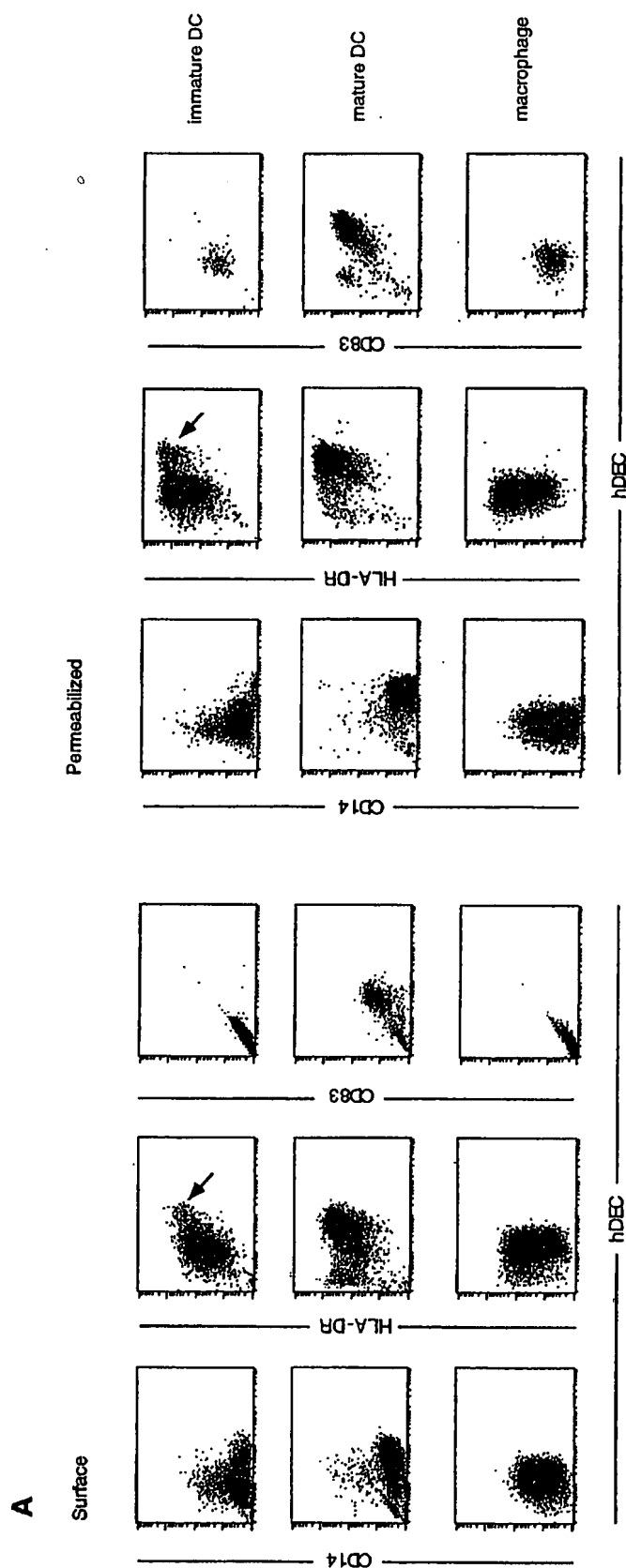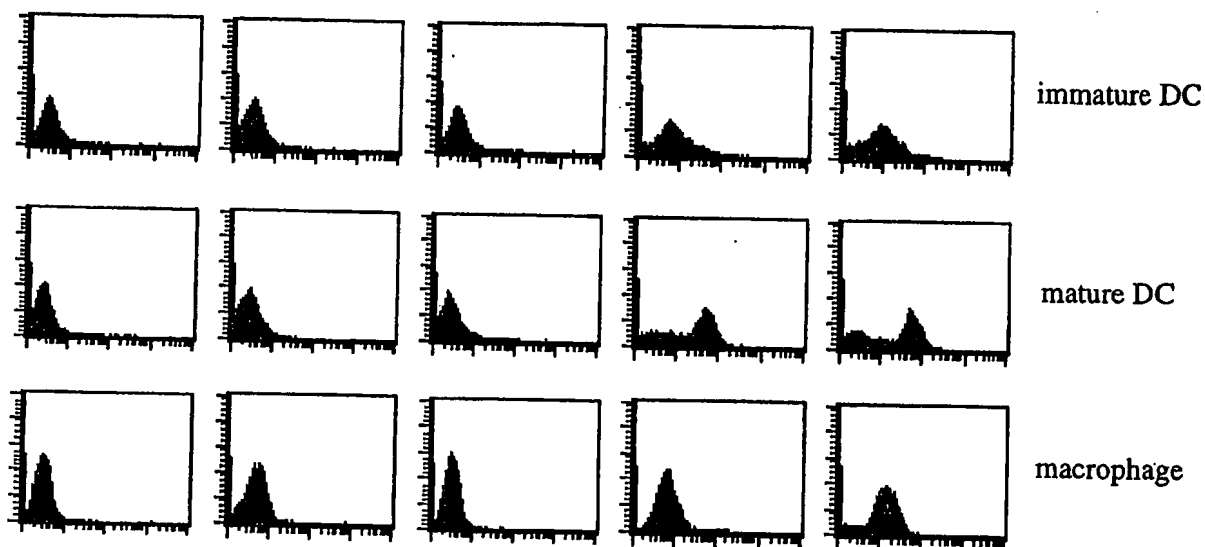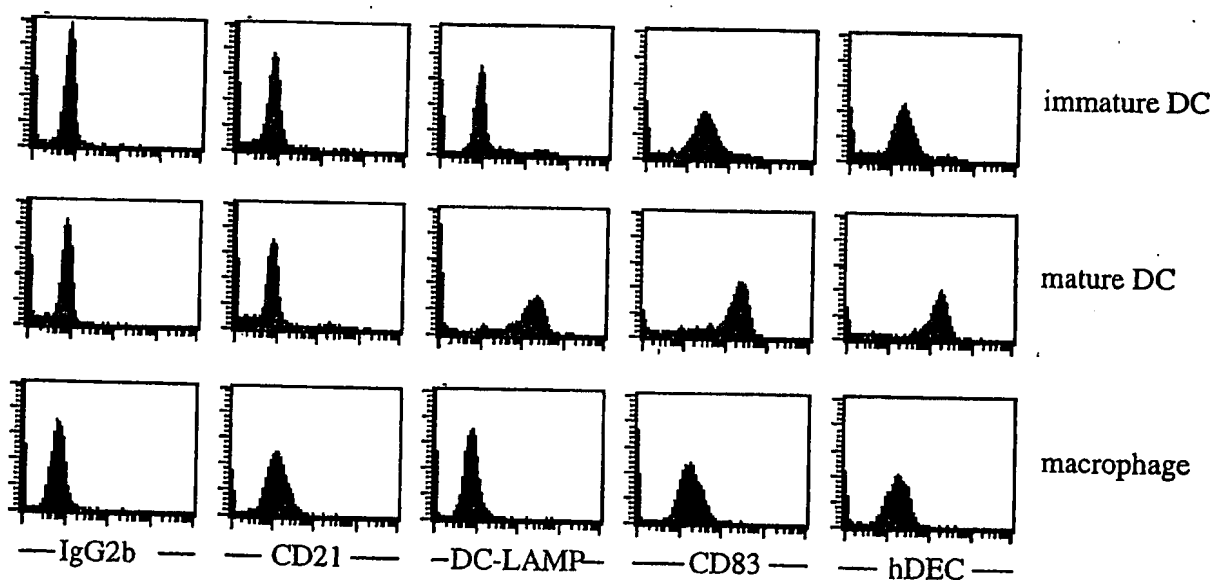
FIGURE 4 (A) DEC-205 is induced in mature DCs. Flow cytometry on live (surface) or saponin-permeabilized monocyte-derived macrophages, immature DCs, and mature DCs. Double staining of cells with MG38, detected by FITC-goat-anti-mouse Ig, and PE-antibodies: PE-CD14, PE-HLA-DR, and PE-CD83. A small subset of mature DEC-205$^+$ HLA-DR$^+$ cells in the immature DC preparation is arrowed.

B

Surface

immature DC

mature DC

macrophage

Permeabilized

immature DC

mature DC

macrophage

—IgG2b —   — CD21 —   –DC-LAMP–   ——CD83 —   — hDEC —

FIGURE 4 (B)   DEC-205 is induced in mature DCs. Flow cytometry on live (surface) or saponin-permeabilized monocyte-derived macrophages, immature DCs, and mature DCs. Histograms of DEC-205, CD83, DC-LAMP, and CD21 expression. Mature DCs, but not immature DCs or macrophages, expressed high levels of DEC-205, CD83 and DC-LAMP.
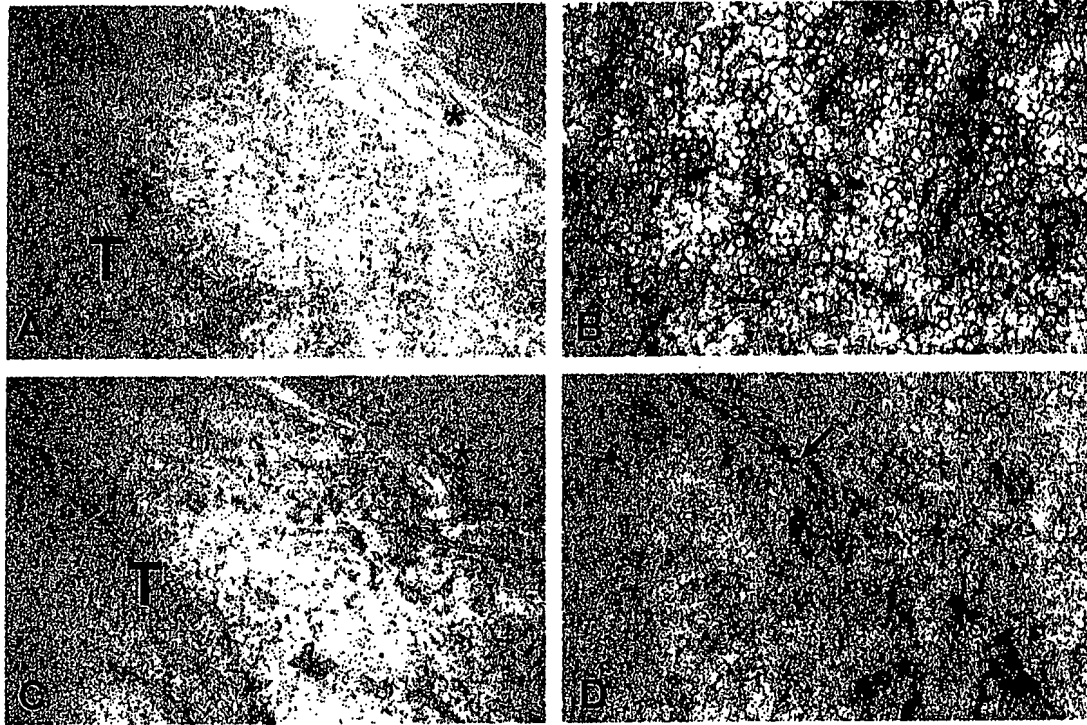
FIGURE 5 Expression of human DEC-205 and MMR in select regions of human tonsil. At low power (A,C), a T cell area (left) is next to a vessel rich (*) nonparenchymal region (right); at high power, a T cell area is shown (B,D). The sections were labeled in brown for CD8 to identify T cells and in blue for DEC-205 (MG 38; panels A,B)or MMR (mAb 3.29; panels C,D). The MMR, when found in the T cell area, is on lymphatic vessels (D, arrow), while in nonparenchymal regions, MMR is found on scattered cells, probably macrophages (C). DEC-205 is found on DCs in the T cell area (B).

ferent sites in lymphoid tissues, and only DEC-205 is abundant on DCs.

DISCUSSION

The availability of monoclonal antibodies to human MMR and DEC-205 now makes it possible to look more closely at the tissue distribution of these homologous, multilectin receptors for adsorptive endocytosis. A single monoclonal has been available to mouse DEC-205 but only polyclonals and antisense RNA probes for the mouse MMR. For human, several monoclonals to the MMR have been produced but none to human DEC-205. We have been able to secure the MG38 monoclonal to human DEC-205 by immunizing DEC-205 knockout mice with the NH_2-terminal region of the recently cloned human DEC-205. The MG38 antibody reacts with the immunogen produced from DEC-205 cDNA, it

precipitates a 205 kD polypeptide from metabolically labeled mature DCs, and it stains the thymic cortical epithelium much like the rat anti-mouse DEC-205 antibody. On isolated human blood cells, the expression of DEC-205 is similar to what is known for mouse DEC-205 [5, 7, 17]. The extent of analysis in the human system is still limited, but DEC-205 is expressed on DCs derived from blood monocytes, while PBMCs stain weakly or not at all with MG38. The presence of weak FACS signals in other leukocytes is in keeping with the fact that our cDNA was derived from a human lymphocyte library.

In tissue sections of tonsil and thymus, DEC-205 and MMR are expressed on different cells. As in the mouse, MG38 stains large dendritic profiles in the T cell areas of tonsil and the medulla of the thymus, as well as the thymic epithelium. In contrast, the MMR is found on scattered macrophages in the trabeculae or septae of both organs, and on efferent lymphatic endothelium in the tonsil. Recently [18], it has been reported that the mouse MMR is often expressed on lymphatic vessels in mouse lymphoid tissues, but not in the T cell areas, a known site for the localization of DCs. Two other studies have shown that a polyclonal antibody to the mouse MMR also stains sinusoidal lining cells in lymph node and liver, but does not stain DCs in the T cell areas [18, 19]. In sum, even though MMR and DEC-205 are homologous in structure and expressed on monocyte-derived
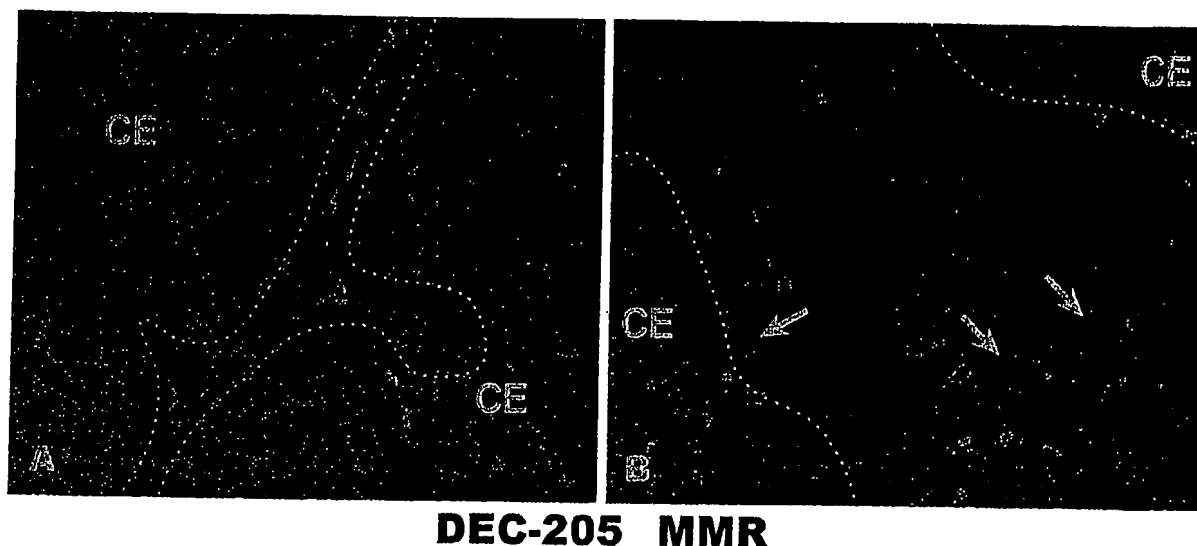
DEC-205 MMR

FIGURE 6 Expression of DEC-205 (red) and MMR (green) in the thymic cortex (A) and medulla (B) using two color indirect immunofluoresence and deconvolution of 0.5 μ sections (see Materials and Methods). A septum in the thymic cortex (A) and the thymic medulla (B), are outlined with white dots. The two receptors are expressed in different cells: scattered, green macrophages for the MMR, and the cortical epithelium and medullary DCs (arrows) for DEC-205.

DCs, the tissue distribution of these two endocytosis receptors is quite different.

Because both the MMR and DEC-205 are found on DCs produced *in vitro* from human monocytes with GM-CSF and IL-4, it is possible that both are involved in the presentation of foreign antigens. Recently, it has been shown that blood derived mouse monocytes can capture particles in a peripheral extravascular site and then enter the lymph and T cell areas of the draining lymph node to become DCs [20]. Monoclonals are not yet available to verify the expression of mouse MMR and DEC-205 on monocyte-derived DCs *in vivo*. However, in the steady state, the MMR and DEC-205 are expressed in different sites in lymphoid tissues and may have distinct functions. We speculate that a biological role of the MMR in sinusoidal endothelium is to clear self proteins that have been altered to expose mannosyl residues. In contrast, DEC-205 on thymic epithelium and DCs in the thymic medulla and T cell areas may function to capture self proteins for thymic and peripheral selection events.

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